**Aggregation and Gelation Properties of Preheated Whey Protein and Pectin Mixtures at
pH 1.0-4.0**

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Abstract

The objective of this work was to understand aggregation and gelation properties of mixtures with preheated whey protein (hWP) and pectin at 1:0-5:1 mass ratios quickly acidified to pH 1.0-4.0 with HCl under agitation. Aggregation properties were studied by measuring turbidity and zeta-potential of hWP-pectin mixtures at pH 1.0-4.0. Gelation properties were characterized for **visual inspection of gel formation** in vials and small strain oscillation tests. Temperature and time sweeps, also with addition of 5 mM N-ethylmaleimide or 10% urea, were used in rheological tests to understand molecular forces causing gelation. The quickly-acidified gels had less homogenous microstructures and lower strengths than those using slow acidification enabled with glucono-delta-lactone **and without agitation**. hWP, pectin, and their mixtures had different gelation properties at pH 1.0-4.0. At the studied conditions, **pectin neutralized positive charges of hWP, induced gelation at pH 1.5-3.0, and changed properties of hWP gels. hWP gelled at pH 3.5-4.0, while the hWP-pectin mixtures did not**. Gelation of hWP alone and its mixture with pectin involved different molecular interactions. Both physical forces (hydrophobic attraction and hydrogen bonds) and disulfide bonds were important to the gelation of hWP at pH 3.5-4.0, while physical forces dominated the gelation of hWP-pectin mixtures, with electrostatic attraction between the two polyelectrolytes playing a significant role. The findings suggest the gelation properties of hWP-pectin mixtures at pH 1.0-4.0 are dependent on the biopolymer ratio and **composition**.

Keywords: preheated whey protein, pectin, aggregation, gelation, rheology

1. Introduction

Food biopolymers, i.e. proteins, polysaccharides, and their mixtures, can increase viscosity and induce the enhanced or sustained satiety (Solah et al., 2010). Understanding aggregation and gelation properties of protein-polysaccharide mixtures at physical, chemical, and biochemical conditions of the stomach may facilitate the formulation of food products that can deliver quality before and during consumption and increase viscosity after ingestion. For this purpose, whey protein (WP) is a good example to formulate beverages, not only because of its high contents of essential amino acids, bland flavor, good digestibility, and health-promoting benefits (Kinsella & Morr, 1984) but also its aggregation properties after preheating. Preheated whey protein (hWP) remains well dispersed at neutral pH with a low viscosity due to the strong electrostatic repulsion, but aggregates after acidification due to the insignificance of the weakened electrostatic repulsion when compared to hydrophobic attraction (Bryant & McClements, 1998).

WP is a mixture of globular proteins including β -lactoglobulin (β -Lg), α -lactalbumin, and lesser amounts of bovine serum albumin, and immunoglobulins (De Wit, 1981). β -Lg, the major component of WP, has an isoelectric point (pI) at about pH 5.1 (Bryant & McClements) and dominates the overall gelation properties of WP (Mulvihill & Kinsella, 1987). Thermal gelation of WP isolate (WPI) and β -Lg has been studied extensively. Thermal denaturation of WP induces the exposure of initially embedded hydrophobic amino acids and sulfhydryl groups that are responsible for aggregation and network formation (Bryant & McClements). Acid-induced gelation of hWP at room temperature is well-known (Alting et al., 2002). Preheating WP at a pH way above the pI and a low ionic strength forms soluble aggregates

between non-polar and sulfhydryl groups exposed after thermal denaturation (Alting et al., 2002; De Wit, 1981). The subsequent acidification toward the pI of WP reduces the number of net negative charges, and the weakened electrostatic repulsion results in the aggregation and possible gelation at ambient temperature (Alting et al., 2002). Anionic polyelectrolytes, such as pectin, can be added to facilitate acid-induced gelation of hWP because the two groups of biopolymers become oppositely charged once pH is lowered to be below the protein pI (Norton & Frith, 2001; de Jong et al., 2009; Zhang, Hsieh, & Vardhanabhuti, 2014; Zhang & Vardhanabhuti, 2014).

Acid-induced gelation of hWP has been studied at pH 6.0-8.0 (Mleko & Foegeding, 2000) and 5.0 (Alting, Hamer, de Kruif, & Visschers, 2003; Alting et al., 2004). Gelation properties of hWP and polysaccharide mixtures were reported at pH 4.0-5.6 (de Jong et al., 2009) and 4.7 (Zhang et al., 2014; Zhang & Vardhanabhuti, 2014). To study fundamental gelation properties, these studies applied glucono-delta-lactone (GDL) that is gradually hydrolyzed to lower pH in several hours without disrupting network formation. However, gastric conditions are quite complicated. The pH is about 1.2-2.5 in an empty stomach of healthy adults and can increase to 4.5-5.8 after ingesting foods, followed by gradual reduction to a pH lower than 3.1 after mixing an increasing amount of secreted gastric juice (Kong & Singh, 2008). When a neutral hWP dispersion is first mixed with gastric juice, the pH is expected to change quickly (relative to solid foods) and the overall pH is dependent on the ratio of the two liquids. The movement in gastric conditions is another factor that can disrupt food structures. Although the overall movement in the stomach is not very intense (Norton et al., 2014), pharmaceutical scientists observed the need to include beads to simulate the digestion of drug

products, which indicates the involvement of local mixing (Kong & Singh, 2008). Additionally, digestive enzymes can change the properties of mixtures during the progress of gastric digestion. These complexities suggest there is not a constant set of physical and chemical conditions that can be used to simulate the dynamics of gastric digestion. Therefore, the above studies applying slow acidification without mixing are insufficient to understand the properties of hWP-pectin mixtures after ingestion.

The objective of the present work was to understand aggregation and gelation characteristics of hWP-pectin mixtures after quick acidification to pH 1.0-4.0 with agitation. This pH range is expected in the stomach during meal time (Mattioli et al., 1990; Kong & Singh, 2008), and we hypothesize that hWP can form gels at this pH range when present at a high enough protein concentration and the gelation properties can be further controlled by pectin and calcium ions. Quick acidification is used to understand properties upon mixing a neutral hWP dispersion and gastric juice. Constant agitation is needed to quickly acidify hWP-pectin mixtures to pH 1.0-4.0 because of the tendency of hWP aggregation below about pH 5.5. Agitation also is expected in the stomach, with contraction forces estimated to be between 0.2 and 1.89 N (Norton et al., 2014). Aggregation properties were studied for turbidity and zeta-potential at low (0.45% w/v) protein concentrations, while gelation properties were studied at 6.3% w/v hWP. Although data from these two sets of samples cannot be directly correlated, properties of diluted samples can provide physical insights about gelation mechanisms at high hWP concentrations. Formation of gels in vials was visually inspected for samples with various pectin-to-WP mass ratios, and sodium chloride or calcium chloride was added to study the effect of electrostatic interactions on gelation. The

microstructure of gels was studied using confocal laser scanning microscopy (CLSM). To understand the role of hydrogen bonds, disulfide bonds, and hydrophobic interactions on gelation, dynamic rheological tests were conducted at isothermal conditions and during temperature ramps, with and without urea or N-ethylmaleimide (NEM). Temperature ramps were used to facilitate the understanding of the significance of physical forces such as hydrogen bonds and hydrophobic interactions on the structures. The aggregation and gelation properties of hWP-pectin mixtures at the studied conditions may assist the understanding of the initial stage of gastric digestion and the designing of structures to control digestion properties.

2. Materials and methods

2.1. Materials

WPI powder was obtained from Hilmar Ingredients, Inc. (Hilmar, CA). The product contained 91.0% w/w protein, 0.2% w/w lactose, 0.2% w/w fat, 4.0% w/w moisture, and 1.0% w/w ash according to the manufacturer. Low-methoxyl pectin from citrus peel with an esterification degree of 32% and a galacturonic acid content of above 74% (Lot # SLBF4758V) was purchased from Sigma-Aldrich Corp. (St. Louis, MO). Urea (99% purity) and NEM (99% purity) were purchased from Acros Organic (Morris Plains, NJ). Unless stated otherwise, all chemicals were of analytical grade.

2.2. Preparation of stock solutions

Fifty grams of WPI powder was dissolved in 500 mL deionized water and adjusted to a total volume of 550 mL and a protein concentration of about 9% w/v. Protein solutions

were adjusted to pH 7.0 using 5.0 and 1.0 M NaOH and hydrated overnight at 4 °C to prepare “native” WP (nWP) samples. nWP solutions were heated for 30 min in a water bath after reaching the set temperature of 80 °C and immediately cooled in an ice/water bath to prepare hWP samples. Both nWP and hWP stock solutions were stored at 4 °C before following studies. The hWP as prepared is expected to be close-to completely denatured, as heating 10% WPI solution at pH 6.8 for 10 min at 80 °C resulted in 90.0% denaturation (Haque et al., 2013).

Pectin stock solution was prepared by dissolving 4.5 g of pectin powder in 100 mL deionized water and stirring on a hot plate with a surface temperature set at 80 °C until no visible insoluble substance. NaCl and CaCl₂ stock solutions were prepared in deionized water at a 5.0 and 2.0 M concentration, respectively.

2.3. Aggregation properties

The pectin stock solution and deionized water were added to the WP stock solution to overall concentrations of 0.045% w/v pectin and 0.45% w/v nWP or hWP. After mixing, samples were adjusted to pH 1.0-4.0 using 1.0 M HCl, and the absorbance at 600 nm (Abs_{600}) was measured within 1 h using a UV/Vis spectrophotometer (model Biomate 5, Thermo Electron Corporation, Woburn, MA). Averages of four sample replicates were reported. Additional samples were prepared for 0.45% w/v hWP and 0.0225, 0.045 and 0.09% w/v pectin, corresponding to respective WP: pectin mass ratios of 20:1, 10:1, and 5:1. To study the influence of ionic strength, the NaCl or CaCl₂ stock solution was pipetted into the solutions with hWP to a final NaCl concentration of 25, 50 or 100 mM or a CaCl₂ concentration of 10 or 20 mM. For samples with NaCl or CaCl₂, the volume of salt stock

solution was deducted from the volume of deionized water during the initial mixing of pectin and WP stock solutions. Averages of two replicates were reported.

2.4. Zeta-potential of dispersions

The zeta-potential of dispersions with pectin, hWP, and their mixtures was measured using a Zetasizer Nano instrument (Malvern Instruments Ltd., Malvern, UK). All measurements were carried out at 21 °C, and averages of triplicates were reported.

2.5. Gelation properties

The pectin stock solution and an appropriate amount of deionized water were added to the hWP stock solution with 9.0% hWP. The mixtures contained 6.3% w/v hWP and 0.315, 0.63, or 1.26% w/v pectin, corresponding to WP: pectin mass ratios of 20:1, 10:1, and 5:1, respectively. The mixtures were then adjusted to pH 1.0-4.0 using 6.0 and 1.0 M HCl under agitation at 700-1100 rpm on a stir plate. The acidification was completed in about 1 min for each sample. Because it was difficult to measure the volume of viscous samples and the acid volume was negligible when compared to the sample volume, the volume of final samples was not adjusted with water. To study the influence of ionic strength, the salt stock solutions were added to reach a NaCl concentration of 25, 50 and 100 mM or a CaCl₂ concentration of 5 and 10 mM in the mixture with hWP and pectin at a mass ratio of 10:1 before pH adjustment. As in Section 2.3, the volume of salt stock solutions was deducted from the volume of deionized water during mixing the WPI and pectin stock solutions. Samples right after acidification were still flowable, and 2 mL of each sample was pipetted into a scintillated glass vial, incubated at room temperature (20 °C) for 1 h, and photographed after inverting vials.

Small strain oscillatory rheology was also applied to study gelation properties. Tests were performed using an AR2000 rheometer (TA Instruments, New Castle, DE) with a concentric cylinder geometry (30 mm in the stator diameter, 28 mm in the rotor diameter, 42 mm in the rotor height). Samples were also added with 10% w/v urea or 5 mM NEM, stirred on a magnetic stir plate at 500 rpm until no visible insoluble particulates, and adjusted to pH 1.0-4.0 as above. The samples after pH adjustment were still pourable and were immediately transferred into the rheometer sample cup. Samples were subjected to a time sweep over 60 min at 37 °C and a subsequent temperature ramp from 37 to 80 °C at a heating rate of 5 °C/min. An oscillation frequency of 1 Hz and a strain amplitude of 0.1% were used in tests to be within the limit of linear viscoelastic regime. To compare with slowly-acidified gels, a neutral sample with 6.3% hWP and 0.2 g GDL/g protein (Eissa & Khan, 2005) was prepared and studied for an 8-h time sweep at identical oscillation conditions. The mixture after incubation for 8 h had a final pH of 4.0. In order to reduce the mixture pH to 4.0 in 1 h, a large amount of GDL/g protein is needed, but the increased ionic strength may change protein aggregation properties. Three sample replicates were tested and averages were reported.

2.6. Confocal laser scanning microscopy (CLSM)

Fourteen milliliters of the 9.0% w/v hWP stock solution were stained with 20 µL of an aqueous solution with 0.2% w/v Rhodamine B. The stained hWP was stirred in the dark for 1 h, mixed with deionized water and/or the pectin stock solution, adjusted for pH, and allowed to form gels as described in Section 2.3. The gels were placed at an area of about 20 mm × 20 mm on slides, and microstructures of gels were studied on a Leica TCS SP2

microscope and a 63× objective lens (Leica Microsystems Inc., Heidelberg, Germany). The excitation wavelength was 543 nm for Rhodamine B (Salcedo et al., 1999). The emission from 562 to 650 nm was collected using a triple dichroic mirror at 488/543/633 nm. Digital images were acquired on a 78 μm × 78 μm area at a 1024 × 1024 pixel resolution.

2.7. Statistical analysis

Statistical analyses were performed using the SAS Enterprise Guide program (version 6.1, SAS Institute, Cary, NC). One-way analysis of variance (ANOVA) was carried out. Differences between pairs of means were compared using Tukey's test. The significance level was set at 0.05.

3. Results and discussion

3.1. Aggregation properties of preheated whey protein and pectin mixtures

3.1.1. Aggregation at a low ionic strength

The aggregation between pectin and WP at pH 3.5-8.0 is a result of electrostatic attraction between negatively charged carboxylate groups of pectin and positively charged amino acid residues of WP and has been studied using parameters of zeta-potential, turbidity, and particle size (Jones, Decker, & McClements, 2009; Jones & McClements, 2011). In the present work, the aggregation properties below pH 4.0 were studied using samples with 0.45% w/v protein and measured for Abs_{600} and zeta-potential. An increase in sample turbidity indicates the increase of quantity or dimension of aggregates which are large enough to scatter visible light (Jones, Lesmes, Dubin, & McClements, 2010). Because an excellent correlation between the aggregate size of hWP and optic density at 420 nm has previously

been reported (Ju & Kilara, 1998), the particle size of samples was not reported in the present study.

nWP has a hydrodynamic diameter of 10 nm at neutral acidity, corresponding to transparent samples with low turbidity (Wang et al. 2012). The clear nWP samples with Abs_{600} below 0.05 at pH 1.0-4.0 (Fig. 1A) indicated the absence of protein aggregation at pH below 4.0, which is expected from solubility properties of nWP (Ju & Kilara, 1998).

Solutions with 0.045% w/v pectin were also clear at pH 1.0-4.0. The high Abs_{600} (2.15) of a solution with 0.45% w/v nWP and 0.045% w/v pectin at pH 4.0 indicates that nWP and pectin may have formed large complexes (Jones & McClements, 2010). The mixture was less turbid when pH decreased from 4.0 to 1.0, indicating a reduced extent of aggregation due to the protonation of carboxylate groups of pectin with pKa of pH 3.47 at 20 °C (Holvik & Høiland, 1977).

nWPs are denatured and form **strands** during heating at neutral pH (Marangoni et al., 2000). The strands formed after heating at pH 7.0 with a low ionic strength have a small width (Ikeda & Morris, 2002) and are not as effective as particulate aggregates scattering visible light (Bryant & McClements, 1998). The turbid appearance and high Abs_{600} of hWP at pH 3.5 and 4.0 (Fig. 1A) indicated the aggregation of WP strands due to the weakened electrostatic repulsion (Bryant & McClements, 2008). Below pH 3.5, samples were clear and had Abs_{600} of about 0.15, indicating the net positive charges of hWP were strong enough to prevent aggregation. The overall trend was similar when pectin was added at one-tenth mass of hWP, except for the increased turbidity at pH 2.0-3.0 (Fig. 1A) which indicates the aggregation between pectin and WP strands. The complexation was further verified in Fig.

1B that showed higher Abs_{600} at pH 2.0-4.0 with a higher content of pectin.

Zeta potentials of hWP, pectin, and their mixtures at pH 2.5-4.0 are shown in Fig. 2.

The measurements below pH 2.5 were affected probably by the reaction between acid and metal electrodes in cells and were not shown. The reduced zeta-potential magnitude of hWP

at a higher pH between 2.5 and 4.0 (Fig. 2) agreed with the higher Abs_{600} (Fig. 1A),

indicating the significance of electrostatic repulsion in preventing protein aggregation. The

gradual reduction in the magnitude of negative zeta-potential of pectin at a lowered pH (Fig.

2) is due to the protonation of carboxylate groups of galacturonic acid with a pKa of 3.47 at

20 °C (Holvik & Høiland, 1977). Therefore, the reduced possibility of complexation between

hWP and pectin resulted in smaller Abs_{600} at a lower pH between 1.0 and 4.0 (Fig. 1B). With

an increase of pectin content, the zeta-potential of hWP-pectin mixtures generally decreased,

due to charge neutralization between the two biopolymers, which increased the possibility of

aggregation between complexes and therefore Abs_{600} (Fig. 1B).

3.1.2. Aggregation at increased ionic strengths

Impacts of ionic strength on WP aggregation were studied for 25-100 mM NaCl or

10-20 mM $CaCl_2$. For solutions with 0.45% w/v nWP or 0.45% w/v pectin, no apparent

change in Abs_{600} was observed after salt addition (data not shown), indicating these ionic

conditions are insufficient to cause the significant aggregation of each biopolymer. Fig. 3A

shows the Abs_{600} of dispersions with 0.45% w/v nWP and 0.045% w/v pectin after addition of

NaCl or $CaCl_2$. At pH 3.0-4.0, an increase in ionic strength generally resulted in a decrease in

Abs_{600} , which is expected because the charge screening effects weaken the electrostatic

attraction between WP and pectin and cause the decoupling of complexes (Jones &

McClements, 2011). At pH 1.0-2.5, no significant impacts of ionic strength on Abs_{600} were observed, resulting from the insignificance of complex formation, as discussed previously.

Fig. 3B shows the increases of Abs_{600} for hWP alone samples at pH 3.0 and 3.5 with an increase in ionic strength, indicating that the weakened electrostatic repulsion between hWP induced further aggregation (Bryant & McClements, 1998). The Abs_{600} of the hWP-pectin mixture at a 10:1 mass ratio increased at pH 2.5-3.5, but decreased at pH 2.0 with the increase of ionic strength (Fig. 3C). The phenomenon can be explained by the relative significance of aggregation and decoupling of hWP-pectin complexes. At pH 2.5-3.5, the aggregation dominated in the system and led to an increase in Abs_{600} , while decoupling dominated at pH 2.0 due to the weakened electrostatic attraction between hWP and pectin.

3.2. Visual inspection of gel formation

3.2.1. Gel formation at a low ionic strength

Gels were first evaluated for formation of gels from samples with 6.3% w/v WP by visual inspection (Fig. 4A). All samples were well-dispersed at pH 7.0 but behaved differently after acidification. Solutions with pectin or nWP did not show noticeable changes at pH 1.0-4.0. The mixture with nWP and pectin did not form gels at pH 1.0-4.0 but became more turbid when compared to samples with only one biopolymer. This observation agrees with the turbidity at a low protein concentration (Fig. 1A) and suggests these complexes cannot form a gel network at the studied compositions.

hWP formed gels at pH 3.5 and 4.0 (Fig. 4A) due to a sufficient extent of aggregation (Fig. 1A) (Bryant & McClements, 1998). Aggregation of hWP was limited below pH 3.5 (Fig. 1A) due to the strengthened electrostatic repulsion (Fig. 2), corresponding to fluid samples

(Fig. 4A). When hWP was mixed with pectin at a mass ratio of 20:1, gels were observed at pH 2.0-3.5 and pH 1.0 (Fig. 4A). When the hWP: pectin mass ratios were 10:1 and 5:1, the gelation pH range shifted to pH 1.0-3.0 and 1.0-2.5, respectively (Fig. 4A). Below pH 4.0, pectin neutralizes positive charges of hWP and facilitates aggregation (Fig. 1B and 2), and a larger amount of pectin facilitates aggregation at pH 2.0-4.0 (Fig. 1B), which explains the shifting in gelation pH of mixtures with an increase of the pectin content. hWP formed gels at pH 1.0 regardless of pectin content (Fig. 4A). The conformation of hWP at this extreme pH is a possible reason of gelation, but the exact mechanism is to be studied.

No gelation but precipitation occurred at pH 4.0, 3.5-4.0, and 3.0-4.0 when the hWP: pectin mass ratio was 1:20, 1:10, and 1:5, respectively. The hWP-pectin mixture has a low magnitude of zeta-potential around pH 4.0 (Fig. 2), which could result in quick aggregation to form large and dense structures (Weinbreck, 2004) that can result in precipitation rather than gelation (Kallala, Jullien, & Cabane, 1992). At a lower pH below 4.0, the complexes of hWP and pectin can have less compact structures (Weinbreck, 2004) due to moderate intra- and intermolecular electrostatic repulsion, which increases the effective volume of biopolymers to favor gelation.

3.2.2. Gel formation at increased ionic strengths

Fig. 4B shows the influence of ionic strength on gelation of mixtures with 6.3% w/v hWP and 0.63% w/v pectin. Addition of NaCl appeared to have weakened the structure at pH 3.0 and 1.5, while gels were still observed at pH 1.0-3.0 after addition of 5 and 10 mM CaCl_2 . As presented previously, NaCl can decouple complexes or facilitate the aggregation of hWP and complexes by screening electrostatic attraction or weakening inter-particle repulsion,

respectively. At pH 3.0, the mixture of hWP and pectin has a smaller positive zeta-potential than at a lower pH (Fig. 2), and the weakened electrostatic repulsion by NaCl may cause the quickened aggregation to weaken structure. At pH 1.5, the charge screening effect of NaCl can reduce the aggregation between protein and pectin with a low extent of protonated carboxylate groups to reduce the possibility of gelation (Fig. 4B). At pH 2.0 and 2.5, these negative effects of NaCl on gelation may not be significant enough to disrupt networks (Fig. 4B). For CaCl_2 treatments, despite the increase of ionic strength, the ability of divalent calcium ions to cross-link anionic low-methoxyl pectin can strengthen the gel network at pH 1.0-3.0 (Kohn, 1987). Observations in Figure 4 indicate the possibility to control gelation properties of hWP at pH 1.0-4.0 using an appropriate hWP: pectin mass ratio and an increased concentration of CaCl_2 .

3.3. Microstructures of gels

Gels with hWP and pectin at a mass ratio of 10:1 adjusted to pH 3.0 and 2.5 and hWP gels at pH 4.0 and 3.5 were studied in CLSM, with images shown in Fig. 5. Red areas represent protein matrices stained by Rhodamine B, and black areas are devoid of protein. Irregular structures with void areas were observed for hWP at pH 3.5 and 4.0 (Fig. 5A and 5B). In previous CLSM studies, hWP acidified using GDL **without agitation** generally had a uniform structure (Alting et al., 2003; Zhang et al., 2014), which is a result from the homogeneous distribution of GDL and slow acidification without **agitation**. In our study, gels were prepared by quick acidification with agitation, which results in an instantaneous regional decrease of the pH (Alting et al., 2004) and heterogeneous structures that can be disrupted and reformed.

Irregular structures were also observed in hWP-pectin gels at pH 2.5 (Fig. 5C) and 3.0 (Fig. 5D). Compared to hWP only samples (Fig. 5A and 5B), the hWP-pectin mixture at pH 2.5 and 3.0 appeared to have formed denser and smaller aggregates (Fig. 5D). At pH 3.0 (Fig. 5D), both islands and small aggregates were observed, with void space throughout the sample. In a previous study (de Jong & van de Velde, 2007), microscopic phase separation was observed in CLSM images of WP-polysaccharide mixture gels with a continuous protein network and a discontinuous serum phase, and sharp phase boundaries were observed at pH 4.8 when a charge density of polysaccharides was below 0.7, including high methoxyl pectin with an esterification degree of 71%. In contrast, microscopic phase separation was not observed in CLSM images of polysaccharides with a charge density above 0.7, including low methoxyl pectin with an esterification degree of 37% (de Jong & van de Velde, 2007). Pectin used in the present study had an esterification degree of 32%, and no microscopic phase separation was observed.

3.4. Rheological properties of gels

Rheological properties were used to investigate gel structures and formation mechanisms. Fig. 6 shows the storage modulus (G') of hWP at pH 3.5 and 4.0 during incubation at 37 °C. The initial G' of hWP at pH 3.5 (Fig. 6A) was much lower than that of the gel at pH 4.0 (Fig. 6B), which agrees with the higher hardness of hWP gels at a pH closer to 5 (Cavallieri et al., 2007). The G' of hWP at pH 4.0 during incubation for 60 min increased to about 450 Pa (Fig. 6B). The phase angle (δ) gradually decreased from about 30° to 15° at pH 3.5 (supplementary Fig. 7A), indicating the increase of solid-like properties during incubation. The δ of hWP at pH 4.0 was less than 15° and also gradually decreased

(supplementary Fig. 7B), indicating the formation of a more solid-like gel than at pH 3.5. In contrast, the G' of hWP acidified by GDL without agitation was about one-order of magnitude higher (~4,700 Pa after 60-min incubation at 37 °C, Fig. 8) than the quickly acidified sample (Fig. 6B). In addition to the absence of structure disruption, the possibility of forming disulfide bonds during slow acidification by GDL **without agitation** (Monahan et al., 1995) can result in a much stronger gel than that acidified quickly.

To investigate molecular interactions in gels, NEM or urea was added to inhibit the formation of disulfide bonds (Alting et al., 2003) or weaken hydrophobic attraction and hydrogen bonds (Lapanje, 1978), respectively. The addition of NEM lowered G' and increased δ of hWP gels at pH 3.5 and 4.0 during time sweeps at 37 °C and heating from 37 to 80 °C (Fig. 6, Fig. 7). The results indicate the formation of disulfide bonds at pH 3.5 and 4.0 and their significance to the formation of gel networks. After addition of urea, hWP at pH 3.5 had a very small G' (Fig. 6A) and fluctuating δ (Fig. 7A). The G' of hWP at pH 4.0 with urea was close to 0 Pa at the beginning of time sweep and was always smaller than that of the treatment with NEM (Fig. 6B). The increase in G' of hWP with urea at pH 4.0 during time sweep may be due to the formation of disulfide bonds. Additionally, the smaller δ of the pH 4.0 sample with urea (<20 °) than that at pH 3.5 (above 35°, Fig. 7A vs. 7B) suggests the more solid-like properties of the former.

During temperature ramps of the hWP samples at pH 4.0 (Fig. 6D), G' decreased at elevated temperatures until about 65 °C, followed by slight increase at higher temperatures, while δ increased slightly but remained to be smaller than 15° (Fig. 7D). The initial decrease of G' can be contributed by the disruption of hydrogen bonds (Bryant & McClements, 1998),

while the later increase of G' can result from continued formation of disulfide bonds and strengthened hydrophobic interactions at increased temperatures (Vardhanabhuti et al., 2001). However, samples after addition of NEM or urea showed insignificant changes of G' (Fig. 6D) during temperature ramps. The observations were similar at pH 3.5 (Fig. 6C) that had much smaller G' than at pH 4.0 in the entire temperature range. These results showed the significance of both chemical (disulfide bonds) and physical forces (hydrogen bonds and hydrophobic attraction) in the network formation of hWP at pH 3.5 and 4.0.

The G' of mixtures with hWP and pectin was overall smaller at pH 3.0 (Fig. 9B) than at pH 2.5 (Fig. 9A), while δ showed the opposite trend (Fig. 10A,B). The opposite trends of G' and δ at pH 3.0 and 2.5 indicate they are two different types of gels, with the former possibly being more brittle. The stronger gel at pH 2.5 agreed with the less open and compact structures of hWP-pectin mixture than at pH 3.0 observed in CLSM (Figure 5), as presented previously. No noticeable decrease in G' and δ of hWP-pectin gels was observed after addition of NEM (Fig. 9A,B and 10A,B), indicating disulfide bonds are insignificant for hWP-pectin gels at pH 2.5 and 3.0. This can be caused by the reduced reactivity of sulfhydryl groups at a lower pH (Monahan et al., 1995) and additional hindrance on disulfide bond formation after coverage of hWP surface by pectin. After addition of urea, the G' of hWP-pectin mixtures at pH 2.5 and 3.0 was reduced to about 20 Pa (Fig. 9A,B), while δ increased (Fig. 10A,B), indicating the weakening of gel strength. The G' of hWP-pectin mixtures at pH 2.5 and 3.0 was significantly higher than the close-to-zero of the initial G' for samples with hWP only at pH 3.5 and 4.0 (Fig. 6). Therefore, hydrogen bonds and hydrophobic attraction are only partially responsible for the gel formation of hWP-pectin

mixtures. Considering the loss of gelation properties at pH 3.0 after addition of a sufficient amount (100 mM) of NaCl (Fig. 4B), electrostatic attraction between hWP and pectin is significant to gelation of the mixture.

During temperature ramps, G' of hWP-pectin samples at both pH 2.5 and 3.0 decreased from 37 to 60 °C, followed by increasing at higher temperatures (Fig. 9C,D), while δ of the samples increased from 17 to 25 ° and 15 to 20 °, respectively (Fig. 10C,D). As above, the decrease of G' below 60 °C may have resulted from the weakened hydrogen bonding, while the increase of G' above 60 °C may have resulted from the strengthened hydrophobic interactions. Samples with NEM showed no significant difference in both G' and δ at pH 2.5 and 3.0, which supports the previous speculation about the insignificance of disulfide bonds. Samples with urea at pH 2.5 and 3.0 had lower G' and higher δ than samples without urea and NEM, and the no increase in G' during heating indicates no significant formation of new bonds.

4. Conclusions

Gelation properties of hWP at pH 1.0-4.0 after quick acidification with agitation were affected by pectin. Quickly-acidified hWP gels with agitation had different structures and properties from the GDL-induced gels without agitation. Agitation during pH adjustment and sample preparation reduced the strength and homogeneity of hWP gels, and quick acidification limited formation of disulfide bonds. Pectin neutralized positive charges of hWP, induced gelation at pH 1.5-3.0, and changed properties of hWP gels. At a pH around 3.5 and 4.0, hWP formed a gel while hWP-pectin did not. Both physical (hydrophobic attraction and

hydrogen bonds) forces and disulfide bonds were important to the gelation of hWP at pH 3.5 and 4.0, while physical forces dominated the gelation of hWP-pectin mixture at pH 2.5 and 3.0, with electrostatic attraction between the two polyelectrolytes playing a significant role. Bridging pectin by divalent calcium ions further changed the gel properties. Findings from the present study shows the possibility to control aggregation and gelation properties of neutral dispersions of hWP-pectin mixtures after quick acidification to pH 1.0-4.0 by controlling their mass ratio and adopting cationic calcium ions. The understanding of the complete gastric digestion profile of hWP-pectin mixtures however will require the incorporation of digestive enzymes in a chamber that simulates the hydrodynamics and pH profile expected during the gastric digestion.

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Figure captions

Figure 1. Absorbance at 600 nm (Abs_{600}) of (A) dispersions with 0.045% w/v pectin, 0.45% w/v “native” whey protein (nWP) or preheated whey protein (hWP), and their mixtures at a WP: pectin mass ratio of 10:1; (B) dispersions with 0.45% hWP and pectin at WP: pectin mass ratios of 1:0, 20:1, 10:1, and 5:1. Error bars are standard deviations ($n = 4$).

Figure 2. Zeta-potentials of dispersions with 0.45% w/v pectin, 0.45% w/v preheated whey protein (hWP), and their mixtures at a WP: pectin mass ratio of 20:1, 10:1, or 5:1. Error bars are standard deviations ($n = 3$).

Figure 3. Absorbance at 600 nm (Abs_{600}) of dispersions with (A) “native” whey protein (nWP) and pectin, (B) preheated whey protein (hWP), and (C) hWP and pectin, after addition of various concentrations of NaCl or $CaCl_2$. WP content was 0.45% w/v in all treatments, and the WP: pectin mass ratio was 10:1. Error bars are standard deviations ($n = 2$).

Figure 4. Photographs of samples with (A) pectin (0.63% w/v), “native” whey protein (nWP) or preheated whey protein (hWP), and their mixtures at different WP: pectin mass ratios, and (B) mixtures with an hWP: pectin mass ratio of 10:1 supplemented with various concentrations of NaCl or $CaCl_2$. WP content was 6.3% w/v in all treatments.

Figure 5. CLSM images of (A) preheated whey protein (hWP) at pH 3.5, (B) hWP at pH 4.0, (C) hWP and pectin at pH 2.5, and (D) hWP and pectin at pH 3.0. hWP (stained by Rhodamine B) and pectin concentrations were 6.3% and 0.63% w/v, respectively. Bar = 10 μm .

Figure 6. Storage modulus (G') of samples with 6.3% w/v preheated whey protein (hWP) adjusted to pH 3.5 (A and C) or 4.0 (B and D) during a time sweep (A and B) at 37 °C and a

subsequent temperature ramp (C and D) from 37 to 80 °C at 5 °C/min. Samples were also added with 10% w/v urea or 5 mM NEM. Averages from three parallel tests are presented.

Figure 7. Phase angle of samples with 6.3% w/v preheated whey protein (hWP) adjusted to pH 3.5 (A and C) or 4.0 (B and D) during a time sweep (A and B) at 37 °C and a subsequent temperature ramp (C and D) from 37 to 80 °C at 5 °C/min. Samples were also added with 10% w/v urea or 5 mM NEM. Averages from three parallel tests are presented.

Figure 8. G' , G'' , and phase angle of mixtures with 6.3% w/v preheated whey protein (hWP) and 1.24% w/v GDL during a time sweep at 37 °C.

Figure 9. Storage modulus (G') of mixtures with 6.3% w/v preheated whey protein (hWP) and 0.63% w/v pectin adjusted to pH 2.5 (A and C) or 3.0 (B and D) during a time sweep at 37 °C (A and B) and a subsequent temperature ramp from 37 to 80 °C at 5 °C/min (C and D). Samples were also added with 10% w/v urea or 5 mM NEM. Averages from three parallel tests are presented.

Figure 10. Phase angle of mixtures with 6.3% w/v preheated whey protein (hWP) and 0.63% w/v pectin adjusted to pH 2.5 (A and C) or 3.0 (B and D) during a time sweep at 37 °C (A and B) and a subsequent temperature ramp from 37 to 80 °C at 5 °C/min (C and D). Samples were also added with 10% w/v urea or 5 mM NEM. Averages from three parallel tests are presented.

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Figure 5.

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Graphical abstract